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David L. Parker

**PATENT**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
Lopez-Berestein et al.

Serial No.: 09/982,113

Filed: October 17, 2001

For: A METHOD TO INCORPORATE N-(4-HYDROXYPHENYL) RETINAMIDE IN LIPOSOMES

Group Art Unit: 1615

Examiner: Kishore, Gollamudi S.

Atty. Dkt. No.: UTSC:660US

# APPEAL BRIEF

**MS Appeal Briefs**
Commissioner for Patents
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Sir:

Appellants hereby submit an original and three copies of this Appeal Brief to the Board of Patent Appeals and Interferences in response to the final Office Action dated February 20, 2004. The Notice of Appeal is filed concurrently herewith.

The fee for filing the Notice of Appeal is $165, and the fee for filing this Appeal Brief is $165; a check is enclosed. If the check is inadvertently omitted, or the amount is insufficient, or should any additional fees under 37 C.F.R. §§ 1.16 to 1.21 be required for any reason relating to the enclosed materials, or should an overpayment be included herein, the Commissioner is authorized to deduct or credit said fees from or to Fulbright & Jaworski L.L.P. Account No.: 50-1212/UTSC:660US.

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## I. REAL PARTY IN INTEREST

The real party in interest is the assignee, Board of Regents, The University of Texas System, Austin, Texas.

## II. RELATED APPEALS AND INTERFERENCES

There are no interferences or appeals for related cases.

## III. STATUS OF THE CLAIMS

Claims 54-141 are pending, of which claims 61-130 are withdrawn. Therefore, claims 54-60 and 131-141 are under active examination.

As explained below, Appellants attempted to amend base claim 54 to focus the present appeal on one of the two alternative embodiments presented by that claim (the "water-containing" embodiment). However, the Examiner refused to enter that narrowing amendment. Therefore, Appellants are proceeding with the present appeal only with respect to dependent claims that present the water-containing embodiment, claims 138-141. It is Appellants'

intention, if successful in the present appeal, to introduce amendments into base claim 54 that are consistent with the water-containing embodiment reflected by appealed claims 138-141.

A copy of the claims on appeal, as well as the other pending and withdrawn claims, is attached as Appendix A.

## IV. STATUS OF AMENDMENTS

Appellants sought an amendment after final to focus the present appeal on the water-containing embodiments. The Examiner refused to enter this amendment, stating that "the limitations of soybean oil and water are now deleted from claim 54, yet dependent claim 141 recites this limitation." This statement is incorrect in that the after-final amendment did not seek to remove the word "water" from claim 54, it only sought the removal of the words "one or more of soybean oil and", which amendment would have effectively introduced the limitations of claim 138 into claim 54. This was actually stated in the response to final, wherein Appellants stated that:

> Independent claim 54 has been amended and is now directed specifically to the use of 4HPR lipid compositions wherein the lipid composition comprises DMPC and water. Claims 133 and 138 have been cancelled and the dependencies of claims 134 and 139 revised accordingly.
>
> Applicants are amending independent claim 54 in order to prosecute the claimed subspecies of allowance/appeal. Applicants reserve the right to proceed with claims directed to the additional subject matter in future continuing applications. If the pending claims are passed to allowance, Applicants would propose to cancel withdrawn claims 61-119 and 130, or authorize an examiner's amendment, in order to place the case into condition for allowance.

The Examiner was subsequently contacted by phone and Appellant's representative attempted to explain the appropriateness of the amendment, but the Examiner again refused to enter the amendment.

## V. SUMMARY OF THE INVENTION

The invention that is the subject of the present appeal is directed to the treatment of cancer using a compound known as N-(4-hydroxyphenyl) retinamide or a derivative thereof (hereinafter collectively "4-HPR") that is formulated in a composition that includes the phospholipid DMPC and water, as well as possibly other elements such as soybean oil. The subject matter of the present appeal is reflected generally by dependent claim 138, which can be depicted in independent form as follows:

> 138. A method of treating a subject having cancer, comprising administering to said individual a therapeutically effective amount of a composition comprising N-(4-hydroxyphenyl) retinamide, or a derivative thereof, encapsulated in a lipid material, wherein said lipid material comprises dimyristoyl phosphatidylcholine (DMPC) and water.

Dependent claims 139 and 140 make reference to specific percentages and ranges of water, whereas claim 141 refers to the inclusion of DMPC, water and soybean oil.

## VI. ISSUES ON APPEAL

The issues addressed in this appeal include:

a) Whether the subject matter of claims 138-141 is obvious over the combination of Mehta, US 5,811,119 (Exhibit 1) in view of Ulukaya (Exhibit 2);

b) Whether the subject matter of claims 138-141 is obvious over the combination of Mehta in view of Minton, US 5,008,291 (Exhibit 3) or Zeligs, US 6,093,706 (Exhibit 4), or vice versa.

## VII. GROUPING OF THE CLAIMS

The claims are to be considered separately, and separate arguments are presented hereinbelow.

## VIII. ARGUMENT

### A. The rejection of claims 138-141 over Mehta in view of Ulukaya

#### 1. Summary of Rejection

The Action rejects claims 138-141 over the combination of Mehta in view of Ulukaya, taking the position that Mehta teaches the use of liposomal retinoids comprising soybean oil and DMPC in the treatment of cancer. Ulukaya is cited as teaching that 4-HPR is a retinoid known to be useful in treating cancer.

The Final Action included no reference to any teaching in either Mehta or Ulukaya that concerns the inclusion of water in the liposomes of Mehta. When this pointed out in Appellants' response to final, the Advisory Action stated merely that "applicant's arguments that Mehta's liposomes do not have water are not found to be persuasive since liposomal bilayer formation occurs only after hydration with water." The Examiner provided no teaching to support this conclusion which, for the reasons stated below, is incorrect.

#### 2. Appellants' Remarks

*a) Substantial evidence required to uphold the examiner's position*

Findings of fact and conclusions of law by the U.S. Patent and Trademark Office must be made in accordance with the Administrative Procedure Act, 5 U.S.C. § 706(A), (E), 1994. *Dickinson v. Zurko*, 527 U.S. 150, 158 (1999). Moreover, the Federal Circuit has held that findings of fact by the Board of Patent Appeals and Interferences must be supported by "substantial evidence" within the record. *In re Gartside*, 203 F.3d 1305, 1315 (Fed. Cir. 2000). In *In re*

*Gartside*, the Federal Circuit stated that "the 'substantial evidence' standard asks whether a reasonable fact finder could have arrived at the agency's decision." *Id.* at 1312.

Accordingly, it necessarily follows that an Examiner's position on Appeal must be supported by "substantial evidence" within the record in order to be upheld by the Board of Patent Appeals and Interferences.

*b) The standard for obviousness*

In order to establish a *prima facie* case of obviousness, three basic criteria must be met: (1) there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings; (2) there must be a reasonable expectation of success; and (3) the prior art reference (or references when combined) must teach or suggest all the claim limitations. *Manual of Patent Examining Procedure* § 2142. Moreover, the teaching or suggestion to make the claimed combination and the reasonable expectation of success must both be found in the prior art, and not based on Applicant's disclosure. *In re Vaeck*, 947 F.2d 488, 20 U.S.P.Q. 2d 1438 (Fed Cir. 1991). When "the motivation to combine the teachings of the references is not immediately apparent, it is the duty of the examiner to explain why the combination of the teachings is proper." MPEP § 2142.

*c) No Proper Prima Facie Rejection Made*

It is Appellant's position that the Examiner has failed to make out a *prima facie* obviousness rejection over the references, alone or in combination, which have not been shown to teach or suggest each of the elements of the claims. The principal reference, Mehta, fails to teach or suggest the use of DMPC and water to form its liposomes. Mehta's Example 1 describes the preparation of liposomal-all trans-retinoic acid ("L-RA"). In the paragraph beginning at col 7, line 54 of Mehta, it is described that the retinoic acid ("RA") is comprised in

t-butanol, and that the butanol-solubilized RA is then added to the "dried lipid film" to form the liposomes. It is specifically stated that only butanol, ***not*** butanol + water, was used to form the liposomal retinoid. Thus, there is no teaching here that water or an aqueous solution is added during the liposomal formulation step.

The Examiner apparently relies upon the statement in Mehta which concerns "reconstitution" of already-formed "liposomal retinoic acid" in an aqueous solution (see, *e.g.*, col. 7, ln 66, to col. 8, ln 3). The Examiner has not shown that resuspending already-formed liposomes in an aqueous solution results in the introduction of water into the lipid layer – in fact, it does not! Resuspending already-formed liposomal/drug in an aqueous results in water going into the interior of the liposome, not into the lipid bilayer. It is noted that the claims require that the "lipid material" used to form the liposomes comprise water. In contrast to the liposomes of Mehta, the present liposomes actually incorporate water in the lipid bilayer by virtue of it's presence in the starting butanol. As explained below and exemplified in the present specification, inclusion of water in the lipid material at the time of formation of the liposome has significant advantages over the Mehta approach of not including water in the lipid material

In contrast, Applicants' specification discloses, for example in Example 1 and Table 2 (page 83; attached as Exhibit 5 hereto), that liposomal 4-HPR can be formed using DMPC and water, or DMPC + water + soybean oil ("SO"), and, as explained in more detail below, that such water containing formulations have surprisingly higher encapsulation efficiencies, at least in the context of 4-HPR, than do 4-HPR lipid formulations prepared without the inclusion of water.

*d) The secondary reference does not cure the defect*

None of the secondary references contains a teaching that remedies the deficiencies in Mehta with respect to the inclusion of water in the DMPC phospholipid composition used to form liposomes. Ulukaya contains no disclosure that Applicants can find that suggests a

liposomal formulation, and the Examiner appears to concede this and simply relies on supposed advantages of 4-HPR *per se*. However, it is unclear how this fact alone provides a suggestion to provide the 4-HPR or water in the lipid formulation of Mehta. Indeed, there is evidence in Ulukaya that teaches away from the combination: Ulukaya teaches that 4-HPR has properties that distinguish it from naturally occurring retinoids, including the fact that it apparently exerts its clinical effects by a different pathway from classical retinoids. This fact suggests that 4-HPR has *different* physicochemical and/or biological properties from classical retinoids, which immediately brings into question whether one of ordinary skill would have an expectation that this very different retinoid could be practiced in the context of the teachings of Mehta. We think not. Furthermore, with the known advantages described in Ulukaya, we question whether one of skill would be motivated to try to modify it in any way. Again, we think not.

e) *Claims 139 and 140*

The subject matter of claims 139 and 140 are separately patentable. Claim 139 is directed to a composition wherein the lipid material comprises from 1 to 10% water, and claim 140 is directed to a composition wherein the lipid material comprises about 10% water. The Examiner has not even attempted to make a *prima facie* rejection of the subject matter of these claims. As noted below in the secondary considerations section, such formulations have surprisingly high 4-HPR encapsulation efficiency, particularly when compared to liposomes prepared without water as taught by Mehta.

*f) Evidence of Secondary Considerations*

As noted above, there exists here strong evidence to support a conclusion of non-obviousness. Applicants' specification discloses, in Example 1 and Table 2 (page 83; attached as Exhibit 5 hereto), that liposomal 4-HPR can be formed using DMPC and water, or DMPC + water + soybean oil ("SO"). Here, the water in included in the t-butanol that is employed for

solubilizing the lipids and 4-HPR to form liposomes. Importantly, the encapsulation efficiency achieved by the present inventors for 4-HPR using water or water + SO was consistently very high: 81.5% with DMPC + water and ranging from 77.5% up to 96.4% with DMPC + water + SO. This was in direct contrast to the much poorer 60% encapsulation efficiency achieved without the inclusion of water, which is reflective of the approach taught by Mehta.

*g) Claims 138-141*

The data set forth in Table 2 demonstrates that formulations made with DMPC + water have a surprisingly higher 4-HPR encapsulation efficiency than formulations made with DMPC and no water (81.5% vs. 60.0%). Similarly, it is shown that increasingly higher amounts of water provide an increasingly higher incorporation efficiency (77.5%, 81.5% and 87.5% for lower amounts of water and 88.3%, 92.8% and 96.4% for higher amounts of water). This is particularly relevant to the patentability of claims 138-140.

Furthermore, the data demonstrates that the inclusion of all three of DMPC + soybean oil + water is even more preferred than either DMPC + water alone, or DMPC alone (average 88.5% vs. 81.5% vs. 60.0%). This is particularly relevant to the patentability of claim 141.

**B. The rejection of claims 138-141 over Mehta in view of Minton or Zeligs**

**1. Summary of Rejection**

The Final Action next rejects claims, including claims 138-141, as obvious over Mehta in view of Zeligs. The rejection is essentially as set forth above for the foregoing rejection, with the exception that the secondary references of Minton and Zeligs are relied upon. Minton is said to teach 4-HPR in the treatment of cancer and is also said to teach the use of sustained or continuous release formulations (col. 13, lns 17-18). Zeligs is also said to teach 4-HPR in the treatment of cancer and is said to teach administration in the form of liposomes (col. 6, ln 60).

## 2. Appellants' Remarks

First of all, Appellants incorporate by reference all of the foregoing comments with respect to the rejection over Mehta in view of Ulukaya. Further with respect to the two additional references of Minton and Zeligs, Appellants note that neither of these references appear to teach or suggest the use of water in the lipids used to form liposomal 4-HPR and thus do not appear to address the shortcomings noted above.

The Minton reference is said to teach sustained or continuous release formulations, but it is hard to see how this disclosure is relevant to DMPC/SO/water formulations of 4-HPR and the Examiner has not provided any explanation in this regard. On the contrary, Minton simply teaches that one can prepare sustained release formulations of the 4-HPR and calcium glucarate. However, it is hard to imagine how the preparation of a sustained release formulation of these two drugs would lead one of skill in the art to Mehta – as noted above, Mehta is concerned with formulations having reduced toxicity and appears to teach that one can administer liposomal retinoids for longer periods of time without toxicity (see, *e.g.*, col. 4). Perhaps Appellants have missed the teachings that the Examiner is relying upon. So, if the Examiner is aware of some teaching in Mehta that its liposomal retinoid formulations are for the purpose of providing a sustained release formulation, the Examiner is requested to identify the teaching relied upon on the record. (Applicants have on-line searched the Mehta patent for the word "sustained" without success, and have only found the word "continuous" in relation to continuous therapy with retinoids as opposed to teaching that the lipid formulations provide this benefit.)

Zeligs is the one reference that does refer generically to liposomal formulations of DHEA and retinoids such as 4-HPR, but again, there is no basis for combining this teaching with Mehta *per se* to arrive at the presently claimed invention. In particular, it is noted that Zeligs is primarily concerned with topical therapy and topical compositions for the treatment of skin

disorders and for protection against UV light. There is some disclosure that concerns parenteral administration – indeed, liposomal formulations are only mentioned in the context of systemic administration (col. 6, ln 60). While no specific indications for liposomal formulations *per se* are given by Zeligs (see col. 6, ln 60), there is disclosure that systemic administration is to "prevent" the "recurrence" of squamous cell carcinoma. Furthermore, there is no disclosure that would, in the Applicants' opinion, suggest to one of skill in the art to select and use a DMPC/SO/water formulations – certainly a very general disclosure such as Zeligs (which only appears to mention the word "liposome" once) cannot render each and every cancer therapeutic methods suing lipid formulations obvious.

## IX. CONCLUSION

Appellants have provided arguments that overcome the pending rejections. Appellants respectfully submit that the Final Official Action's conclusions that the claims should be rejected are unwarranted. It is therefore requested that the Board overturn the Final Action's rejections.

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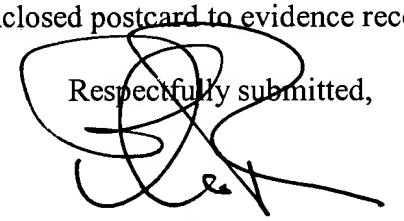
Respectfully submitted,



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## APPENDIX A

1. – 53. (Cancelled)

54. (Previously presented) A method of treating a subject having cancer, comprising administering to said individual a therapeutically effective amount of a composition comprising N-(4-hydroxyphenyl) retamide, or a derivative thereof, encapsulated in a lipid material, wherein said lipid material comprises dimyristoyl phosphatidylcholine (DMPC) and one or more of soybean oil (SO) and water.

55. (Original) The method of claim 54, wherein said dimyristoyl phosphatidylcholine and soybean oil comprise a ratio of greater than 80:20.

56. (Original) The method of claim 54, wherein said composition is comprised in a pharmaceutically acceptable aqueous medium.

57. (Original) The method of claim 54, wherein said method further comprises administering at least one additional therapeutic agent to said individual.

58. (Previously presented) The method of claim 57, wherein said agent is comprised in said composition.

59. (Original) The method of claim 57, wherein said additional therapeutic agent comprises at least one anticancer agent.

60. (Original) The method of claim 59, wherein the anticancer agent is chemotherapy agent, a radiotherapy agent, an immune therapy agent, a genetic therapy agent, a hormonal therapy agent, a biological agent, an additional retinoid or a retinoid derivative.

61. (Withdrawn) A method for increasing growth inhibitory effects of fenretinide on a cell comprising providing to a cell, in combination with fenretinide, one or more agents that increases the level of nitric oxide (NO) in said cell.

62. (Withdrawn) The method of claim 61, wherein said cell is a tumor cell.

63. (Withdrawn) The method of claim 62, wherein said tumor cell is a breast cancer cell.

64. (Withdrawn) The method of claim 63, wherein the breast cancer cell is an estrogen receptor (ER)-positive cell.

65. (Withdrawn) The method of claim 63, wherein the breast cancer cell is an estrogen receptor (ER)-negative cell.

66. (Withdrawn) The method of claim 61, wherein fenretinide is provided before the one or more agents.

67. (Withdrawn) The method of claim 61, wherein fenretinide is provided at the same time as the one or more agents.

68. (Withdrawn) The method of claim 61, wherein fenretinide is provided after the one or more agents.

69. (Withdrawn) The method of claim 61, wherein fenretinide is provided more than once.

70. (Withdrawn) The method of claim 69, wherein fenretinide is provided daily for three months with monthly three-day interruptions.

71. (Withdrawn) The method of claim 61, wherein said agent is provided more than once.

72. (Withdrawn) The method of claim 61, wherein said agent is a nucleic acid.

73. (Withdrawn) The method of claim 72, wherein said nucleic acid is an expression construct encoding iNOS, interferon-γ or herceptin.

74. (Withdrawn) The method of claim 61, wherein said agent is a protein.

75. (Withdrawn) The method of claim 74, wherein said protein is iNOS, interferon-γ or herceptin.

76. (Withdrawn) The method of claim 61, wherein said agent is a chemopharmaceutical.

77. (Withdrawn) The method of claim 76, wherein said agent is cyclosporin A.

78. (Withdrawn) The method of claim 62, wherein said cell tumor cell is a patient.

79. (Withdrawn) The method of claim 78, wherein said cell tumor cell is part of a tumor mass in said patient.

80. (Withdrawn) The method claim 78, wherein providing comprises direct administration to said tumor cell.

81. (Withdrawn) The method of claim 61, further comprising providing to said cell an additional anti-cancer therapy.

82. (Withdrawn) The method of claim 81, wherein said additional anti-cancer therapy is radiation.

83. (Withdrawn) The method of claim 81, wherein said additional anti-cancer therapy is a distinct chemotherapy.

84. (Withdrawn) The method of claim 81, wherein said additional anti-cancer therapy is a distinct gene therapy.

85. (Withdrawn) The method of claim 81, wherein said additional anti-cancer therapy is immunotherapy.

86. (Withdrawn) The method of claim 81, wherein said additional anti-cancer therapy is hormonal therapy.

87. (Withdrawn) The method of claim 61, wherein fenretinide is provided in an amount sufficient to achieve an intracellular concentration of 0.1 μm.

88. (Withdrawn) The method of claim 61, wherein fenretinide is provided in an amount sufficient to achieve an intracellular concentration of 0.5 μm.

89. (Withdrawn) The method of claim 61, wherein fenretinide is provided in an amount sufficient to achieve an intracellular concentration of 1.0 μm.

90. (Withdrawn) The method of claim 61, wherein said cell is killed.

91. (Withdrawn) A method for treating cancer in a subject comprising providing to said subject, in combination, fenretinide and one or more agents that increases the level of nitric oxide (NO) in cancer cells in said subject.

92. (Withdrawn) The method of claim 91, wherein said cancer is a breast cancer.

93. (Withdrawn) The method of claim 92, wherein cells of said breast cancer are estrogen receptor (ER)-positive.

94. (Withdrawn) The method of claim 92, wherein cells of said breast cancer are estrogen receptor (ER)-negative.

95. (Withdrawn) The method of claim 91, wherein fenretinide is provided before the one or more agents.

96. (Withdrawn) The method of claim 91, wherein fenretinide is provided at the same time as the one or more agents.

97. (Withdrawn) The method of claim 91, wherein fenretinide is provided after the one or more agents.

98. (Withdrawn) The method of claim 91, wherein fenretinide is provided more than once.

99. (Withdrawn) The method of claim 98, wherein fenretinide is provided daily for three months with monthly three-day interruptions.

100. (Withdrawn) The method of claim 91, wherein said agent is provided more than once.

101. (Withdrawn) The method of claim 91, wherein said agent is a nucleic acid.

102. (Withdrawn) The method of claim 101, wherein said nucleic acid is an expression construct encoding iNOS, interferon-γ or herceptin.

103. (Withdrawn) The method of claim 91, wherein said agent is a protein.

104. (Withdrawn) The method of claim 103, wherein said protein is iNOS, interferon-γ or herceptin.

105. (Withdrawn) The method of claim 91, wherein said agent is a chemopharmaceutical.

106. (Withdrawn) The method of claim 105, wherein said agent is cyclosporin A.

107. (Withdrawn) The method claim 91, wherein providing comprises direct administration to said tumor cell.

108. (Withdrawn) The method of claim 91, further comprising providing to said cell an additional anti-cancer therapy.

109. (Withdrawn) The method of claim 108, wherein said additional anti-cancer therapy is radiation.

110. (Withdrawn) The method of claim 108, wherein said additional anti-cancer therapy is a distinct chemotherapy.

111. (Withdrawn) The method of claim 108, wherein said additional anti-cancer therapy is a distinct gene therapy.

112. (Withdrawn) The method of claim 108, wherein said additional anti-cancer therapy is immunotherapy.

113. (Withdrawn) The method of claim 108, wherein said additional anti-cancer therapy is hormonal therapy.

114. (Withdrawn) The method of claim 91, wherein fenretinide is provided in an amount sufficient to achieve an intracellular concentration in cancer cells of 0.1 μm.

115. (Withdrawn) The method of claim 91, wherein fenretinide is provided in an amount sufficient to achieve an intracellular concentration in cancer cells of 0.5 μm.

116. (Withdrawn) The method of claim 91, wherein fenretinide is provided in an amount sufficient to achieve an intracellular concentration in cancer cells of 1.0 μm.

117. (Withdrawn) The method of claim 91, wherein fenretinide is provided at 10 mg/day.

118. (Withdrawn) The method of claim 91, wherein fenretinide is provided at 100 mg/day.

119. (Withdrawn) The method of claim 91, wherein fenretinide is provided at 200 mg.day.

120. – 129. (Cancelled)

130. (Withdrawn) A method for inhibiting metastasis in a subject having cancer comprising providing to said subject, in combination, fenretinide and one or more agents that increases the level of nitric oxide (NO) in cancer cells in said subject.

131. (Previously presented) The method of claim 54, wherein the composition is administered parenterally to the individual.

132. (Previously presented) The method of claim 54, wherein the composition is administered orally to the individual.

133. (Previously presented) The method of claim 54, wherein the lipid material comprises DMPC and SO.

134. (Previously presented) The method of claim 133, comprising a ratio of 4-HPR, or derivative thereof, to DMPC/SO of from 1:5 to 1:15.

135. (Previously presented) The method of claim 134, wherein the 4-HPR, or derivative thereof, to DMPC/SO ratio is about 1:5 (w/w).

136. (Previously presented) The method of claim 134, wherein the 4-HPR, or derivative thereof, to DMPC/SO ratio is about 1:10 (w/w).

137. (Previously presented) The method of claim 134, wherein the 4-HPR, or derivative thereof, to DMPC/SO ratio is about 1:15 (w/w).

138. (Previously presented) The method of claim 54, wherein the lipid material comprises DMPC and water.

139. (Previously presented) The method of claim 138, wherein the composition comprises from 1 to 10% water.

140. (Previously presented) The method of claim 139, wherein the composition comprises about 10% water.

141. (Previously presented) The method of claim 54, wherein the lipid material comprises DMPC, SO and water.

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LABORATORY – CLINIC INTERFACE

CANCER TREATMENT REVIEWS

# Fenretinide and its relation to cancer

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Retinoids, natural or synthetic substances which have vitamin A activity, have a well-known reputation for their antitumour and differention-inducing activity in vitro and in vivo. More than 1500 retinoids have been tested so far but very few of them have been entered into clinical trials because of their side-effects. All-trans-N-(4-hydroxyphenyl)retinamide (4HPR or fenretinide) is a synthetic retinoid that is reported to have fewer side-effects compared to naturally occurring retinoids such as all-trans retinoic acid (ATRA) and 9-cis retinoic acid. In addition, fenretinide has been shown to induce cell death (apoptosis) even in ATRA-resistant cell lines. Although the mechanism by which fenretinide acts is not entirely known it is considered to be a promising drug and seems to induce apoptosis via different pathway(s) from classical retinoids. In this reveiw, we discuss possible mechanisms of fenretinide action and summarize results of clinical trials.

## INTRODUCTION

It has been known for many years that vitamin A is essential for normal differentiation and maintenance of epithelial tissues. In addition, vitamin A, usually known as retinol, and its derivatives, called retinoids, have an increasing importance as chemopreventive agents through their antiproliferative and/or differentiating effects on certain types of tumours (1). Recently, some retinoids, such as fenretinide, have also been shown to induce cell death by apoptosis in some cancer cell lines (see below), which can be considered as a contribution to their total antitumour effects. However, neither the mechanisms of their chemopreventive effects nor their effects on apoptosis are fully understood.

So far, more than 1500 retinoids have been produced and tested under in-vivo or in-vitro conditions. All-trans-N-(4-hydroxyphenyl)retinamide (4HPR or fenretinide), first produced in the USA in the 1960s, is one such compound (Figure 1). As can be seen from its structure, it is a synthetic amide derived from all-trans-retinoic acid (ATRA). According to the results of various in-vitro cell culture studies, fenretinide seems not only to inhibit the proliferation of cancer cells but also can cause cell death by inducing apoptosis in T lymphoma and T lymphoblastoid leukemia cells (2), human breast cancer cells (3), small cell lung cancer cell lines (4), non-small cell lung cancer cells (5), prostate adenocarcinoma cells (6), head and neck squamous cell carcinoma (SCC) cells (7,8), and melanoma cells (9).

Fenretinide has also been tested in animal models in terms of its antitumour effect *in vivo*. Fenretinide inhibited the proliferation and also induced apoptosis of colon adenoma cells in male F344 rats (10), reduced the yield of carcinogen-induced colon tumours in rats (11,12), induced complete regression of carcinogen-induced first mammary tumours in 22% of animals and partial regression in a further 19% of animals (13), inhibited the induction of mammary adenocarcinomas compared with carcinogen controls in female rats (14), suppressed bladder carcinoma induction by N-butyl-N-(4-hydoxybutyl) nitrosamine (15), exerted chemopreventive effects against exogenous and endogenous rat liver carcinogenesis (16), reduced carcinogen-induced pancreatic adenomas in female hamsters (17), inhibited tumour progression and multiplicity in a two-stage skin cancer model in CD-1 and SENCAR mice (18), reduced naturally occurring skin tumours in ACI/segHapBR rats (19), and if given via the peritoneal cavity rather than by mouth, significantly increased the survival time of mice with ovarian carcinoma (20). In contrast to these beneficial effects, fenretinide did not inhibit the total tumour formation in the lung of female A/J mice in which carcinogenesis was chemically induced by tobacco nitrosamines (21). In addition, there was no protective effect against the induction

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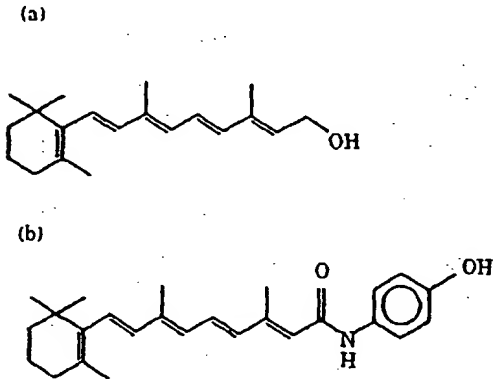


Figure 1 Structures of vitamin A (retinol) (A) and fenretinide (B)

of chemical carcinogenesis of prostate in Wistar-Unilever rats (22). In another study, although fenretinide reduced the incidence of prostate tumors in treated rats compared to controls (27.5 versus 43.2%), the difference was not statistically significant (19). However, fenretinide considerably reduced the development of prostate cancer in the Lobund-Wistar rat model to about one fourth (21 versus 88%) (23).

On the basis of these data, it was decided that fenretinide was a promising candidate for chemoprevention trials. It has therefore been entered into a number of clinical trials in oncology, mainly because of its anticancer properties coupled with lower side effects compared to other retinoids (24,25).

## METABOLISM AND PHARMACOKINETICS OF FENRETINIDE

It seems that fenretinide has significantly different properties when compared with other retinoids in terms of its mode of storage and plasma half-life. The main difference is the absence of hepatic accumulation of fenretinide, implying reduced liver toxicity. Fenretinide did not cause any detectable increase in hepatic retinoid levels in rats after oral administration (26) and in the same study, fenretinide was also found to be selectively accumulated in the mammary glands, which makes it an attractive agent for chemoprevention of breast cancer. The absence of hepatic accumulation was also confirmed by another study although transient increases in liver function tests occurred after each dose (27). In this study, the terminal plasma half-life of fenretinide was found to be 12 hours which is much longer than its natural analog, ATRA. This finding may also make fenretinide more preferable. In contrast, in another study (28) performed on both rats and mice, liver was the second organ in both species accumulating the highest concentrations of fenretinide, with the bladder and mammary glands the first and third highest respectively. In a trial of fenretinide in women who had been operated on for breast cancer, a rise in liver enzymes to two to four times higher than normal levels occurred in only seven of 101 patients without clinically significant liver toxicity (29).

Fenretinide is mainly metabolized to a lipophilic compound, N-(4-methoxyphenyl)-all-trans-retinamide (MPR), which is the major circulating fenretinide metabolite, as well as polar retinamides, including HPR-O-glucuronide (30). Polar metabolites are excreted into urine and bile, while the nonpolar metabolite, MPR, accumulates in tissues including fat, prostate, skeletal muscles, liver and intestines. In another study in female mice after an oral treatment with 10 mg/kg, the highest levels of MPR were detected in liver and mammary tissue (31).

Whether or not MPR is an active metabolite seems to be controversial. It was tested as an active substance (32). Moreover, MPR was stated to be equipotent to fenretinide in reversing keratinization of retinoid-deficient hamster trachea *in vitro* (30). Conversely however, MPR was biologically inert in one study (33). In addition, it has recently been reported not to be an active metabolite of fenretinide as it failed to inhibit the growth of fenretinide-resistant UISO-Mel-6 cells, and showed no dose-dependent inhibition of fenretinide-sensitive breast carcinoma and melanoma cell lines (34). It was also suggested by the same authors that although MPR is not an active metabolite of fenretinide, detection of this metabolite in malignant cells might serve as an indirect biomarker for predicting the response of cells to fenretinide because MPR was detected only in cells sensitive to fenretinide. MPR is also a major determinant of fenretinide-induced reductions in plasma insulin-like growth factor-1 (35) and retinol (36).

Some studies have shown that fenretinide may affect retinol metabolism in the liver. For example, it seems to be able to induce the secretion of RBP, retinol binding protein, the protein which carries retinol in the plasma from the liver into the bloodstream, and leads to rapid RBP accumulation in the kidney (37) In another study (38) it was concluded that fenretinide partially blocks the secretion of the retinol-RBP complex from the liver and other tissues, and thus depresses plasma concentrations of both vitamin A and RBP. Interestingly, it was also demonstrated that fenretinide induced liver RBP secretion in vitamin A deficient rats but depressed it in those with adequate levels of vitamin A (39).

It has recently been claimed that fenretinide itself binds to RBP, and thereby induces secretion of RBP in HepG2 cells, and that the secreted fenretinide-RBP

complex has a reduced affinity for TTR (transthyretin). This observation may explain the fenretinide-induced reduction of plasma retinol observed in in-vivo studies (40). Plasma retinol reduction after fenretinide treatment was also observed in human subjects. For example, in one of the chemopreventive trials, fenretinide treatment resulted in reduction in the levels of plasma retinol (41). However, baseline retinol plasma concentrations recovered 1 month after treatment interruption. Retinol levels were also found to have a negative relationship with fenretinide dose (42) This decrease in plasma retinol might be responsible for the improvements observed in patients with actinic keratoses who applied topical fenretinide twice daily for 3 months (43). It has also been suggested that fenretinide may have clinical utility because of its ability to increase the biological half-life of ATRA (44).

## MECHANISM OF BIOLOGICAL ACTIVITY AND APOPTOSIS-INDUCING EFFECT OF FENRETINIDE

The biological activities, including the anti-growth effect, of fenretinide could be mediated by interaction with the nuclear retinoid receptors. However, it is not entirely clear whether fenretinide can in fact activate these receptors. In transactivation assays, it was observed that fenretinide was a potent transactivator with RARγ and a moderate activator with RARβ, but did not interact with RARα and RXRα. Furthermore, optimal receptor activation has been found at fenretinide concentrations which correspond with those required for its activities as a potent growth inhibitor and inducer of apoptosis (45).

Growth inhibition by fenretinide as well as ATRA was stated to be correlated with the induction of the RARβ2 gene in GLC82 (lung adenocarcinoma) and BGC823 (stomach adenocarcinoma) cells. Furthermore, exogenous RARβ2 expression potentiated fenretinide-induced growth inhibition, suggesting that fenretinide acts at least in part via the RARβ receptor (46). In contrast, in one study exposure to fenretinide resulted in the generation of DNA fragmentation with subsequent cell death in both ATRA-positive oestrogen receptor (ER)-positive as well as ATRA-refractory ER-negative breast carcinoma cell lines. Consequently, it was suggested that fenretinide might mediate its biological actions via a novel pathway(s) not involving the classical retinoid receptor pathways (47). Taken together, because of different retinoid receptor expressions in various tissues, the anti-tumour effect of fenretinide may be limited to some tissues. Its mode of action may also be different from that of ATRA. The observed differential responsiveness of a number of haemopoietic cell lines, which are resistant to ATRA, but which respond to fenretinide, also indicates that these compounds may act through different receptors (48). It has been suggested that fenretinide and indeed ATRA, at least in terms of mediating growth inhibition, might act by different mechanisms (49). It has also been demonstrated that contrary to the differentiation-promoting activity of ATRA, fenretinide dramatically suppressed neuroblastoma (NB) cell growth by inducing apoptosis (50). As mentioned above, fenretinide has been shown to induce apoptosis in head and neck SCC cells whereas ATRA did not appear to have any apoptosis-inducing effect on the same cell lines (7). Indeed, it has been suggested that ATRA can promote a semi-apoptosis-resistant status in neutrophils, possibly through the overexpression of the Bcl-2 gene, whose product is known to be an apoptosis-inhibiting protein, while fenretinide can cause apoptosis (51).

As mentioned above, the mechanism of apoptosis induction by fenretinide as well as by some other retinoids is not well understood. One possibility is that fenretinide can enhance the generation of reactive oxygen species which may then be involved in the apoptotic pathway (52). This phenomenon seems to be restricted to fenretinide and is not observed with other retinoids, including ATRA and 9-cis-retinoic acid (9-cis-RA) (52,53). Another possibility is that fenretinide may affect the cell survival signals received via integrins from the extracellular matrix. It is thought that abrogation of cell adhesion by fenretinide through down-modulation of integrin receptors plays a crucial role in the induction of neuroblastoma programmed cell death (54). However, in this case the same effect was also achieved by ATRA. Fenretinide may also affect the cell cytoskeleton. For example, it has been suggested that fenretinide might trigger apoptosis by inducing overall cytoarchitectural changes and specific DNA fragmentation subsequent to increased turnover of the protein actin in HL-60 cells (51).

In general, retinoids are well known for their differentiating effects, but the effect of fenretinide on differentiation has only been observed in a few cases. For example, fenretinide completely reversed keratinization in squamous metaplasia of hamster tracheal organ cultures resulting from vitamin A deficiency (26), while it either did not show (51) or had a poor (44) differentiating effect on the human promyelocytic leukemia cell lines, HL-60 and NB4, respectively. Furthermore, it was also demonstrated that, contrary to the differentiation-promoting activity of ATRA, fenretinide dramatically suppressed neuroblastoma cell growth by inducing programmed cell death (50). However, fenretinide up-regulated the expression of several differentiation markers

(e.g., class 1 HLA, laminin, and β1 integrin chain), and down-regulated expression of molecules associated with tumour progression, including the p185/HER2 oncoprotein, the epidermal growth factor receptor, and the M(r) 67,000 laminin receptor in breast cancer cell lines (55).

HPR can induce the expression of transforming growth factor-β1 (TGF-β1) in association with the induction of apoptosis (56). It may also regulate the activity of certain cell surface receptors. Thus, fenretinide as well as ATRA treatment of esophageal squamous carcinoma cell lines resulted in downregulation of the epidermal growth factor receptor (EGFR) which is known to bind proliferative ligands such as epidermal growth factor and transforming growth factor α (TGF-α) (57).

The fenretinide concentration used may be the critical point at which cells have to decide whether to undergo either cell death by apoptosis and/or necrosis or cytostasis. Moreover, the type of cell, normal or transformed, also seems to be important in this respect. In one study, fenretinide failed to inhibit the growth of some cervical carcinoma cell lines when used at 1 M but when used at 5 or 10 M, it induced apoptosis (58). In contrast, we found that fenretinide at 1 M inhibited the growth of cell line A431, a squamous cell carcinoma cell line (unpublished observations). We also found that the growth of skin fibroblasts was not affected by the same concentration as was used for A431 cells.

## FENRETINIDE AND APOPTOSIS-RELATED GENE PRODUCTS

There may be a relationship between fenretinide and apoptosis-related gene products, such as p53 and the Bcl-2 family. An extensive study on these gene products was carried out on human leukaemic cell lines and although changes in Bcl-2, p53, and c-myc expression were observed in cells treated with HPR, the time-course of these events suggested that fenretinide-triggered apoptosis was not directly controlled by these genes and that ectopic overexpression of Bcl-2 markedly delayed the onset of apoptosis, rather than protecting cells from death by fenretinide (59). A study on human breast cancer cells showed that fenretinide treatment resulted in decreased Bcl-2 mRNA levels but not Bax mRNA levels, and induced apoptosis in both oestrogen receptor-positive and -negative cells (3). However, it was also found that the fenretinide failed to modulate cellular levels of the Bcl-2 and Bax proteins (52). In another study, p53 and pRB were suppressed in response to fenretinide in the androgen-independent human prostatic JCA-1 cells (60).

## SYNERGISTIC COMBINATIONS OF FENRETINIDE WITH ANTI-CANCER DRUGS

Fenretinide has been used in a number of synergistic combinations in order to try to increase its antitumor efficacy on different types of cancer cells. For example, it seemed that fenretinide potentiated the effects of cisplatin in ovarian carcinoma (20,61). It has also been combined with glucarate and this combination therapy inhibited the growth of human mammary tumour cells grown in athymic mice, the growth of rat mammary tumours in germfree rats, and the hormone-independent MTW 9a/R rat mammary tumour (62). Combinations of fenretinide with either tamoxifen or various cytokines also seems promising.

One study (63) concluded that fenretinide induces the expression of TGF β-1 in association with the induction of apoptosis in prostate cancer cells in vitro, and furthermore, fenretinide-induced cytotoxicity was abrogated by the addition of anti- TGF β-1 antibody. It was also reported that the combination of fenretinide (0.1 μM) and tamoxifen (1 μM) or IFN- γ (500 IU/ml) generally had additive or synergistic effects on all the breast cancer cell lines investigated (64). In an in-vivo study (65), a combination of fenretinide with tamoxifen was found to be effective in inhibiting MNU-induced adenocarcinomas in female Sprague-Dawley rats, and the reduction in tumour incidence was greater for this combination than for either agent alone. Combinations of fenretinide and tamoxifen can be safely administered to women (66). This combination was also found to be more effective than treatment with either of the agents alone in inhibiting of growth of human colon cancer cells in vitro (67). Fenretinide may have a synergistic effect with irradiation too. Low dose fenretinide combined with low dose gamma-irradiation seemed to have a synergistic effect on apoptosis, with the number of apoptotic cells increased by more than 30% (68).

## TOXICITY AND CLINICAL TRIALS WITH FENRETINIDE

In Phase I/II trials, fenretinide seemed to be well-tolerated with only minimal or mild toxicity depending on the dose used. There are currently a number of National Cancer Institute (NCI)-sponsored clinical trials still in progress (69). It was previously reported that the recommended dose for chemoprevention trials of HPR orally was 200 mg per day (70).

A Phase I/II trial in women with oestrogen receptor (ER)-positive or progesterone receptor (PR)-positive,

previously untreated metastatic breast cancer was performed to evaluate the combined toxicity of tamoxifen plus fenretinide (71). There were no significant adverse effects on renal, hepatic, hematologic, or lipid values. Nyctalopia, photophobia, cheilitis, and pruritus (typical features of retinoid treatment) were not observed. Improvement or stabilization of disease occurred in 12 of 15 patients. Therefore it was concluded by authors that tamoxifen administered with fenretinide is nontoxic. Phase III trials of tamoxifen versus tamoxifen plus fenretinide are now warranted. Toxicity was mild in another phase II study of fenretinide involving 31 patients with either advanced breast cancer or melanoma (72).

Toxicity and the feasibility of using fenretinide as a chemoprevention agent in men at high risk for prostate cancer has been evaluated in 22 patients. Fenretinide was well-tolerated, and no major toxicity was associated with its use (73). However, because eight patients with negative prestudy biopsies had positive prostate biopsies prior to, or at the time of, their 12th cycle evaluation, the study was closed early.

In one chemopreventive trial, fenretinide was shown to prevent recurrences and the development of new sites of oral leukoplakia. Again the drug was well tolerated (74,75). However, fenretinide was not effective in a Phase II trial in the treatment of myelodysplasia and it was even stated that in some patients fenretinide may enhance leukaemic progression (76). This result may not be all that surprising because fenretinide tends to accumulate especially in solid tissues such as breast, prostate, and, to some extent but not at a toxic level, in the liver.

Another chemopreventive trial aiming at preventing contralateral primaries in women already treated for breast cancer, whose risk is 0.8% per year within 10 years from primary treatment, started in 1987: accrual of patients was closed in 1993 with a total of 2972 patients, and the study is still ongoing (24). The other trial is the basal cell carcinoma study, which started in January 1990. The study is ongoing (77).

## CONCLUSION

It may be concluded from both in-vivo and in-vitro studies that fenretinide is well-tolerated and has some anti-cancer properties, including anti-proliferative and apoptosis-including effects. More clinical trials are obviously warranted but much work remains to be carried out in order to elucidate its mechanism(s) of action.

## REFERENCES


1. Sporn MB, Dunlop NM, Newton DL, Smith JM. Prevention of chemical carcinogenesis by vitamin A and its synthetic analogs (retinoids). *Federation Proceedings* 1976; 35: 1332–1338.
2. Chan LN, Zhang S, Shao J, Waikel R, Thompson EA, Chan TS. N-(4-hydroxyphenyl) retinamide induces apoptosis in T lymphoma and T lymphoblastoid leukemia cells. *Leukemia and Lymphoma* 1997; 25: 271–280.
3. Wang TT, Phang JM. Effect of N-(4-hydroxyphenyl)retinamide on apoptosis in human breast cancer cells. *Cancer Letters* 1996; 107: 65–71.
4. Kalemkerian GP, Slusher R, Ramalingam S, Gadgeel S, Mabry M. Growth inhibition and induction of apoptosis by fenretinide in small-cell lung cancer cell lines. *Journal of the National Cancer Institute* 1995; 87: 1674–1680.
5. Zou CP, Kurie JM, Lotan D, Zou CC, Hong WK, Lotan R. Higher potency of N-(4-hydroxyphenyl)retinamide than all-trans-retinoic acid in induction of apoptosis in non-small cell lung cancer cell lines. *Clinical Cancer Research* 1998; 4: 1345–1355.
6. Igawa M, Tanabe T, Chodak GW, Rukstalis DB. N-(4-hydroxyphenyl) retinamide induces cell cycle specific growth inhibition in PC3 cells. *Prostate* 1994; 24: 299–305.
7. Eicher SA, Lotan R. Differential effects of retinoic acid and N-(4-hydroxyphenyl) retinamide on head and neck squamous cell carcinoma cells. *Laryngoscope* 1996; 106: 1471–1475.
8. Scher RL, Saito W, Dodge RK, Richtsmeier WJ, Fine RL. Fenretinide-induced apoptosis of human head and neck squamous carcinoma cell lines. *Otolaryngology Head and Neck Surgery* 1998; 118: 464–471.
9. Montaldo PG, Pagnan G, Pastorino F, et al. N-(4-hydroxyphenyl) retinamide is cytotoxic to melanoma cells in vitro through induction of programmed cell death. *International Journal of Cancer* 1999; 81: 262–267.
10. Zheng Y, Kramer PM, Lubet RA, Steele VE, Kelloff GJ, Pereira MA. Effect of retinoids on AOM-induced colon cancer in rats: modulation of cell proliferation, apoptosis and aberrant crypt foci. *Carcinogenesis* 1999; 20: 255–260.
11. Zheng Y, Kramer PM, Olson G, Lubet RA, Steele VE, Kelloff GJ, Pereira MA. Prevention by retinoids of azoxymethane-induced tumours and aberrant crypt foci and their modulation of cell proliferation in the colon of rats. *Carcinogenesis* 1997; 18: 2119–2125.
12. Silverman J, Katayama S, Zelenakas K, *et al.* Effects of retinoids on the induction of colon cancer in F344 rats by N-methyl-N-nitrosourea or by 1,2-dimethylhydrazine. *Carcinogenesis* 1981; 2: 1167–1172.
13. Dowlatshahi K, Mehta RG, Thomas CF, Dinger NM and Moon RC. Therapeutic effect of N-(4-hydroxyphenyl)retinamide on N-methyl-N-nitrosourea-induced rat mammary cancer. *Cancer Letters* 1989; 47: 187–192.
14. Moon RC, Kelloff GJ, Detrisac CJ, Steele VE, Thomas CF, Sigman CC. Chemoprevention of MNU-induced mammary tumors in the mature rat by 4-HPR and tamoxifen. *Anticancer Research* 1992; 12: 1147–53.
15. Becci PJ, Thompson JH, Sporn MB, Moor RC. Retinoid inhibition of highly invasive urinary bladder carcinomas induced in mice by N-butyl-N-(4-hydoxybutyl)nitrosamine (OH-BBN). *Proc Am Assoc Cancer Res* 1980; 21: 88.
16. Tamura K, Nakae D, Horiguchi K, *et al.* Inhibition by N-(4-hydroxyphenyl)retinamide and all-trans-retinoic acid of exogenous and endogenous development of putative preneoplastic, glutathione S-transferase placental form-positive lesions in the livers of rats. *Carcinogenesis* 1997; 18: 2133–2141.

17. Birt DF, Sayed S, Davies MH, Pour P. Sex differences in the effects of retinoids on carcinogenesis by N-nitrosobis(2-oxopropyl)amine in Syrian hamsters. *Cancer Letters* 1981; 14: 13–21.
18. McCormick DL, Moon RC. Antipromotional activity of dietary N-(4-hydroxyphenyl)retinamide in two-stage skin tumorigenesis in CD-1 and SENCAR mice. *Cancer Letters* 1986; 31: 133–138.
19. Ohshima M, Ward JM, Wenk ML. Preventive and enhancing effects of retinoids on the development of naturally occurring tumors of skin, prostate gland, and endocrine pancreas in aged male ACI/segHapBR rats. *Journal of the National Cancer Institute* 1985; 74: 517–524.
20. Formelli F, Cleris L. Synthetic retinoid fenretinide is effective against a human ovarian carcinoma xenograft and potentiates cisplatin activity. *Cancer Research* 1993; 53: 5374–5376.
21. Conaway CC, Jiao D, Kelloff GJ, Steele VE, Rivenson A, Chung FL. Chemopreventive potential of fumaric acid, N-acetylcysteine, N-β4-hydroxyphenyl) retinamide and (-carotene for tobacco-nitrosamine-induced lung tumors in A/J mice. *Cancer Letters* 1998; 124: 85–93.
22. McCormick DL, Rao KV, Dooley L, *et al.* Influence of N-methyl-N-nitrosourea, testosterone, and N-(4-hydroxyphenyl)-all-trans-retinamide on prostate cancer induction in Wistar-Unilever rats. *Cancer Research* 1998; 58: 3282–3288.
23. Pollard M, Luckert PH, Sporn MB. Prevention of primary prostate cancer in Lobund-Wistar rats by N-(4-hydroxyphenyl)retinamide. *Cancer Research* 1991; 51(13): 3610–3611.
24. Costa A, De Palo G, Decensi A, *et al.* Retinoids in cancer chemoprevention. Clinical trials with the synthetic analogue fenretinide. [Review] *Annals of the New York Academy of Sciences* 1995; 768: 148–162.
25. Naik HR, Kalemkerian G, Pienta KJ. 4-Hydroxyphenylretinamide in the chemoprevention of cancer [Review]. *Advances in Pharmacology* 1995; 33: 315–347.
26. Moon RC, Thompson HJ, Becci PJ, *et al.* N-(4-hydoxyphenyl)retinamide, a new retinoid for prevention of breast cancer in the rat. *Cancer Research* 1979; 39: 1339–1346.
27. Swanson BN, Zaharevitz DW, Sporn MB. Pharmacokinetics of N-4-hydroxyphenyl-all-trans-retinamide in rats. *Drug Metabolism and Disposition* 1980; 8: 168–172.
28. Hultin TA, May CM, Moon RC. N-(4-hydroxyphenyl)-all-trans-retinamide pharmacokinetics in female rats and mice. *Drug Metabolism and Disposition* 1986; 14: 714–717.
29. Costa A, Malone W, Perloff M, *et al.* Tolerability of the synthetic retinoid fenretinide (HPR). *European Journal of Cancer and Clinical Oncology* 1989; 25: 805–808.
30. Swanson BN, Newton DL, Roller PP, Sporn MB. Biotransformation and biological activity of N-(4-hydroxyphenyl)retinamide derivatives in rodents. *Journal of Pharmacology and Experimental Therapeutics* 1981; 219: 632–7.
31. Hultin TA, Filla MS, McCormick DL. Distribution and metabolism of the retinoid, N-(4-methoxyphenyl)-all-trans-retinamide, the major metabolite of N-(4-hydroxyphenyl)-all-trans-retinamide, in female mice. *Drug Metabolism and Disposition* 1990; 18: 175–179.
32. Kazmi SM, Plante RK, Visconti V, Lau CY. Comparison of N-(4-hydroxyphenyl)retinamide and all-trans-retinoic acid in the regulation of retinoid receptor-mediated gene expression in human breast cancer cell lines. *Cancer Research* 1996; 56: 1056–1062.
33. Jinno H, Steiner MG, Mehta RG, Osborne MP, Telang NT. Inhibition of aberrant proliferation and induction of apoptosis in HER-2/neu oncogene transformed human mammary epithelial cells by N-(4-hydroxyphenyl)retinamide. *Carcinogenesis* 1999; 20: 229–236.
34. Mehta RR, Hawthorne ME, Graves JM, Mehta RG. Metabolism of N-[4-hydroxyphenyl]retinamide (4-HPR) to N-[4-methoxyphenyl]retinamide (4-MPR) may serve as a biomarker for its efficacy against human breast cancer and melanoma cells. *European Journal of Cancer* 1998; 34: 902–907.
35. Torrisi R, Pensa F, Fontana V, Costa A, Decensi A. The metabolite N-4-methoxyphenylretinamide is a major determinant of fenretinide induced decline of plasma insulin-like growth factor-1 [letter]. *European Journal of Cancer* 1995; 31A: 420–421.
36. Decensi A, Formelli F, Torrisi R, Costa A. Breast cancer chemoprevention: studies with 4-HPR alone and in combination with tamoxifen using circulating growth factors as potential surrogate endpoints. *Journal of Cellular Biochemistry Supplement* 1993; 17G: 226–233.
37. Schaffer EM, Ritter SJ, Smith JE. N-(4-hydroxyphenyl)retinamide (fenretinide) induces retinol-binding protein secretion from liver and accumulation in the kidneys in rats. *Journal of Nutrition* 1993; 123: 1497–1503.
38. Smith JE, Lawless DC, Green MH, Moon RC. Secretion of vitamin A and retinol-binding protein into plasma is depressed in rats by N-(4-hydroxyphenyl)retinamide (fenretinide). *Journal of Nutrition* 1992; 122: 1999–2009.
39. Ritter SJ and Smith JE. Retinol-binding protein secretion from the liver of N-(4-hydroxyphenyl) retinamide-treated rats. *Biochimica et Biophysica Acta* 1996; 1290: 157–164.
40. Holven KB, Natarajan V, Gundersen TE, Moskaug JO, Norum KR, Blomhoff R. Secretion of N-(4-hydroxyphenyl) retinamide-retinol-binding protein from liver parenchymal cells: evidence for reduced affinity of the complex for transthyretin. *International Journal of Cancer* 1997; 71: 654–659.
41. Formelli F, Clerici M, Campa T, *et al.* Five-year administration of fenretinide: pharmacokinetics and effects on plasma retinol concentrations. *Journal of Clinical Oncology* 1993; 11: 2036–2042.
42. Formelli F, Carsana R, Costa A, *et al.* Plasma retinol level reduction by the synthetic retinoid fenretinide: a one year follow-up study of breast cancer patients. *Cancer Research* 1989; 49: 6149–6152.
43. Moglia D, Formelli F, Baliva G, *et al.* Effects of topical treatment with fenretinide (4-HPR) and plasma vitamin A levels in patients with actinic keratoses. *Cancer Letters* 1996; 110: 87–91.
44. Taimi M, Breitman TR. N-4-hydroxyphenylretinamide enhances retinoic acid-induced differentiation and retinoylation of proteins in the human acute promyelocytic leukemia cell line, NB4, by a mechanism that may involve inhibition of retinoic acid catabolism. *Biochemical and Biophysical Research Communications* 1997; 232: 432–436.
45. Fanjul AN, Delia D, Pierotti MA, Rideout D, Qiu J, Pfahl M. 4-Hydroxyphenyl retinamide is a highly selective activator of retinoid receptors. *Journal of Biological Chemistry* 1996; 271: 22441–22446.
46. Liu G, Wu M, Levi G. Ferrari N. Inhibition of cancer cell growth by all-trans retinoic acid and its analog N-(4-hydroxyphenyl) retinamide: a possible mechanism of action via regulation of retinoid receptors expression. *International Journal of Cancer* 1998; 78: 248–254.
47. Sheikh MS, Shao ZM, Li XS, *et al.* N-(4-hydroxyphenyl)retinamide (4-HPR)-mediated biological actions involve retinoid receptor-independent pathways in human breast carcinoma. *Carcinogenesis* 1995; 16: 2477–2486.
48. Delia D, Aiello A, Lombardi L, *et al.* N-(4-hydroxyphenyl)retinamide induces apoptosis of malignant hemopoietic cell lines including those unresponsive to retinoic acid. *Cancer Research* 1993; 53: 6036–6041.
49. Brigati C, Ferrari N, Megna M, *et al.* A retinoic acid resistant HL-60 cell clone sensitive to N-(4-hydroxyphenyl) retinamide-mediated clonal growth inhibition. *Leukemia and Lymphoma* 1995; 17: 175–180.
50. Ponzoni M, Bocca P, Chiesa V, *et al.* Differential effects of N-(4-hydroxyphenyl)retinamide and retinoic acid on neuroblastoma cells: apoptosis versus differentiation. *Cancer Research* 1995; 55: 853–861.

51. Dipietrantonio A, Hsieh TC, Wu JM. Differential effects of retinoic acid (RA) and N-(4-hydroxyphenyl) retinamide (4-HPR) on cell growth, induction of differentiation, and changes in p34cdc2, Bcl-2, and actin expression in the human promyelocytic HL-60 leukemic cells. *Biochemical and Biophysical Research Communications* 1996; 224: 837–842.
52. Oridate N, Suzuki S, Higuchi M, Mitchell MF, Hong WK, Lotan R. Involvement of reactive oxygen species in N-(4-hydroxyphenyl) retinamide-induced apoptosis in cervical carcinoma cells. *Journal of the National Cancer Institute* 1997; 89: 1191–1198.
53. Delia D, Aiello A, Meroni L, Nicolini M, Reed JC, Pierotti MA. Role of antioxidants and intracellular free radicals in retinamide-induced cell death. *Carcinogenesis* 1997; 18: 943–948.
54. Rozzo C, Chiesa V, Caridi G, Pagnan G, Ponzoni M. Induction of apoptosis in human neuroblastoma cells by abrogation of integrin-mediated cell adhesion. *International Journal of Cancer* 1997; 70: 688–698.
55. Pellegrini R, Mariotti A, Tagliabue E. *et al.* Modulation of markers associated with tumor aggressiveness in human breast cancer cell lines by N-(4-hydroxyphenyl) retinamide. *Cell Growth and Differentiation* 1995; 6: 863–869.
56. Roberson KM, Penland SN, Padilla GM. *et al.* Fenretinide: induction of apoptosis and endogenous transforming growth factor beta in PC-3 prostate cancer cells. *Cell Growth and Differentiation* 1997; 8: 101–111.
57. Muller A, Nakagawa H, Rustgi AK. Retinoic acid and N-(4-hydroxyphenyl) retinamide suppress growth of esophageal squamous carcinoma cell lines. *Cancer Letters* 1997; 113: 95–101.
58. Oridate N, Lotan D, Mitchell MF, Hong WK, Lotan R. Inhibition of proliferation and induction of apoptosis in cervical carcinoma cells by retinoids: implications for chemoprevention. *Journal of Cellular Biochemistry -Supplement* 1995; 23: 80–86.
59. Delia D, Aiello A, Formelli F. *et al.* Regulation of apoptosis induced by the retinoid N-(4-hydroxyphenyl) retinamide and effect of deregulated Bcl-2. *Blood* 1995; 85: 359–367.
60. Hsieh TC, Ng C, Wu JM. The synthetic retinoid N-(4-hydroxyphenyl) retinamide (4-HPR) exerts antiproliferative and apoptosis-inducing effects in the androgen-independent human prostatic JCA-1 cells. *Biochemistry and Molecular Biology International* 1995; 37: 499–506.
61. Supino R, Crosti M, Clerici M, *et al.* Induction of apoptosis by fenretinide (4HPR) in human ovarian carcinoma cells and its association with retinoic acid receptor expression. *International Journal of Cancer* 1996; 65: 491–497.
62. Webb TE, Abou-Issa H, Stromberg PC, Curley RC Jr, Nguyen MH. Mechanism of growth inhibition of mammary carcinomas by glucarate and the glucarate: retinoid combination. *Anticancer Research* 1993; 13: 2095–2099.
63. Roberson KM, Penland SN, Padilla GM, *et al.* Fenretinide: induction of apoptosis and endogenous transforming growth factor beta in PC-3 prostate cancer cells. *Cell Growth and Differentiation* 1997; 8: 101–111.
64. Coradini D, Biffi A, Pellizzaro C, Pirronello E, Di Fronzo G. Combined effect of tamoxifen or interferon-beta and 4-hydroxyphenylretinamide on the growth of breast cancer cell lines. *Tumour Biology* 1997; 18: 22–29.
65. Moon RC, Kelloff GJ, Detrisac CJ, Steele VE, Thomas CF, Sigman CC. Chemoprevention of MNU-induced mammary tumors in the mature rat by 4-HPR and and tamoxifen. *Anticancer Research* 1992; 12: 1147–1153.
66. Cobleigh MA. Breast cancer and fenretinide, an analogue of vitamin A. [Review]. *Leukemia* 1994; 8 Suppl 3:S59–63.
67. Ziv Y, Gupta MK, Milsom JW, Vladisavljevic A, Brand M, Fazio VW. The effect of tamoxifen and fenretinimide on human colorectal cancer cell lines in vitro. *Anticancer Research* 1994; 14: 2005–2009.
68. Zou C, Wang L, Liebert M, Grossman HB, Lotan R, Wei Q. Combined effect of chemopreventive agent N-(4-hydroxyphenyl) retinamide (4-HPR) and gamma-radiation on bladder cancer cell lines. *International Journal of Oncology* 1998; 13: 1037–1041.
69. Kelloff GJ and Chemoprevention Branch and Agent Development Committee, Division of Cancer Prevention and Control, National Cancer Institute. Clinical development plan: N-(4-hydroxyphenyl) retinamide (4-HPR). *Journal of Cellular Biochemistry* 1994; 20: 176–196.
70. Costa A, Malone W, Perloff M, et al. Tolerability of the synthetic retinoid Fenretinide (HPR). *European Journal of Cancer and Clinical Oncology* 1989; 25: 805–808.
71. Cobleigh MA, Dowlatshahi K, Deutsch TA, *et al.* Phase I/II trial of tamoxifen with or without fenretinide, an analog of vitamin A, in women with metastatic breast cancer. *Journal of Clinical Oncology* 1993; 11: 474–47.
72. Modiano MR, Dalton WS, Lippman SM, Joffe L, Booth AR, Meyskens FL Jr. Phase II study of fenretinide (N-[4-hydroxyphenyl]retinamide) in advanced breast cancer and melanoma. *Investigational New Drugs* 1990; 8: 317–319.
73. Pienta KJ, Esper PS, Zwas F, Krzeminski R, Flaherty LE. Phase II chemoprevention trial of oral fenretinide in patients at risk for adenocarcinoma of the prostate. *American Journal of Clinical Oncology* 1997; 20: 36–39.
74. Costa A, Formelli F, Chiesa F, Decensi A, De Palo G, Veronesi U. Prospects of chemoprevention of human cancers with the synthetic retinoid fenretinide. [Review] *Cancer Research* 1994; 54(7 Suppl): 2032s–2037s.
75. Chuesa F, Tradati N, Marazza M, *et al.* Fenretinide (4-HPR) in chemoprevention of oral leukoplakia. *Journal of Cellular Biochemistry* Supplement 1993; 17F: 255–261.
76. Garewal HS, List A, Meyskens F, Buzaid A, Greenberg B, Katakkar S. Phase II trial of fenretinide [N-(4-hydroxyphenyl) retinamide] in myelodysplasia: possible retinoid-induced disease acceleration. *Leukemia Research* 1989; 13: 339–343.
77. De Palo G, Veronesi U, Marubini E, *et al.* Controlled clinical trials with fenretinide in breast cancer, basal cell carcinoma and oral leukoplakia. [Review] *Journal of Cellular Biochemistry-Supplement* 1995; 22: 11–17

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

## EXAMPLE 1

## PREPARATION OF LIPOSOMAL 4-HPR

To demonstrate the simplified production of a 4-HPR-liposome composition, 4-HPR was mixed with lipid mixture composed of various ratios of dimyristoyl-phosphatidylcholine (DMPC) and soybean oil in tertiarybutyl alcohol-water mixture (Table 2).

**Table 2**
**Incorporation efficiency of 4-HPR in liposomes**

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Composition of liposome** | **4-HPR: Lipid (w/w)** | 1 : 17 | 1 :10 | | | | 1 : 5 | 1 : 15 |
| | **DMPC: soybean oil in lipid mixture** | 1 : 0 | 1 : 0 | 9 : 1 | | 8 : 2 | 9 : 1 | 9 : 1 |
| | **Water in tertiary butyl alcohol** | 0 | 1 | 1 | 10 | 1 | 10 | 10 |
| **Incorporation efficiency (%)** | | 60.0 | 81.5 | 87.5 | 92.8 | 77.5 | 88.3 | 96.4 |

The mixture was frozen in acetone-dry ice bath, and then dried by lyophilzer. It was stored as a powder and resuspended with saline before use.

To determine the incorporation efficiency of 4-HPR into liposomes, liposomal 4-HPR powder was resuspended in saline and any free, unincorporated free 4-HPR was separated by centrifugation at 30,000 xg for 1 hour. Liposomal 4-HPR was collected as a

pellet and washed three times with saline. 4-HPR concentration was determined by absorbance at 345 nm. To remove the interference by liposomal turbidity, absorbance was measured after liposomal 4-HPR before and after separation was diluted with 1:1 mixture of dimethylsulfoxide and water. The incorporation efficiency was calculated by the following equation:

Incorporation efficiency = OD345 (after separation)/OD345 (before separation) X 100%

## EXAMPLE 2

## ANTI-TUMOR ACTIVITY OF LIPOSOMAL 4-HPR

Anti-tumor activity of liposomal 4-HPR was demonstrated in breast cancer cell lines in terms of growth inhibition (Table 3A and 3B). Breast cancer cell lines were plated in 96 well plates in DMEM/F12 medium supplemented with 5% fetal calf serum and incubated with different concentrations of liposomal 4-HPR and free 4-HPR, respectively. At the end of incubation, the growth of cells was determined by the Celltiter 96 Aqueous nonradioactive cell proliferation assay (Promega Corp., Madison, WI) according to the manufacturer's protocol. Growth and viability of cells were determined by MTS assay after treatment with 4-HPR for 3 days (BT-474) and 4 days (MCF7). Growth of treated cells was compared with that of untreated cells cultured under the same conditions, and was expressed as % of untreated cells. Tables 3A and 3B shows the resulting data, which represents at least 3 experiments for each number.

**Table 3A**
**Comparison of the inhibitory effect of free- and liposomal 4-HPR on the growth and viability of MCF-7 cells**

| Concentration of 4-HPR (μM) | Growth and viability (%) after treatment with | |
|---|---|---|
| | Free 4-HPR | Liposomal 4-HPR |
| 0.1 | 106.5±7.8 | 112.7±2.4 |
| 0.9 | 77.5±5.8 | 72.9±2.9 |
| 1.5 | 43.8±5.7 | 49.2±3.6 |
| 1.8 | 33.6±2. | 35.0±2.6 |